

ROSEMARY VOLATILE OIL AS A PRESERVATIVE AGENT IN SOME CANNED MEAT FOODS

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ABSTRACT

This research was aimed to evaluate activity of Rosemary volatile oil and Nisin A in vivo and on *B. cereus* isolated from some canned meat products in vitro. The results showed that the activity of Rosemary volatile oil (2000 µg/ml) and Nisin A (350 µg/ml) attained to 27 and 19 mm inhibitory zone diameter respectively in well diffusion method. The viable plate count from samples of canned meat treated with effective concentration of Rosemary volatile oil and Nisin A were examined. The samples with Rosemary volatile oil was not showed any CFU/g after 9 days of preservation while sample with Nisin A and control observed 49 and 45 CFU/g respectively. In vivo experiment on mice, two weeks after oral dose of Rosemary volatile oil (2000 µg/ml) and Nisin A (350 IU/ml), there were no death. Furthermore, there were no changes in histological sections taken from liver and spleen of mice treated with Rosemary volatile oil while Nisin A have shown changes in liver and spleen weights and gross or histological characteristics. In conclusion, the Rosemary volatile oil have higher antibacterial effect against *B. cereus* compare with using Nisin A which caused more damage to the organs in the liver and spleen, Thus, it can use the volatile oil of this plant as a preservative at the same time for preservation of meat.

Key words: *Bacillus cereus*, meat products, *16S rRNA*, Nisin A and B

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الزيت الطيار لأكليل الجبل كمادة حافظة في بعض اللحوم المعلبة

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المستخلص

تهدف الدراسة إلى تحديد فعالية النياسين A و الزيت الطيار لأكليل الجبل في الجسم الحي لفتران التجارب على بكتريا *B. cereus* المعزولة من اللحوم المعلبة في المختبر. أظهرت النتائج فعالية اكليل الجبل بتركيز (2000 ميكروغرام / مل) والنياسين A (350 ميكروغرام / مل) إذ بلغت 27 و 19 ملم قطر المنطقة المثبطة على التوالي باستخدام طريقة الانتشار بالحفر. تم فحص عدد المستعمرات الحية في عينات اللحم المعلب المعاملة بالتركيز الفعال من الزيت الطيار لنبات اكليل الجبل ومادة النياسين A. لم تظهر العينات التي تحتوي على الزيت الطيار لنبات اكليل الجبل على أي مستعمرة بكتيرية حية في اللحوم بعد 9 أيام من الحفظ بينما لوحظ ان العينة المعاملة بمادة النياسين A والسيطرة كان تعداد المستعمرات الحية فيها 45 و 49 على التوالي. في التجربة المخبرية التي أجريت على الفتران، بعد أسبوعين من تناول جرعة الزيت الطيار لنبات اكليل الجبل (2000 ميكروغرام / مل) والنياسين A (350 وحدة عالمية / مل) ، لم يكن هناك أي وفاة. علاوة على ذلك ، لم تكن هناك تغييرات في المقاطع النسيجية المأخوذة من الكبد والطحال من الفتران التي عولجت بالزيت الطيار لنبات اكليل الجبل بينما أظهرت مادة النياسين A تغييرًا في أوزان الكبد والطحال ، والخصائص الإجمالية أو النسيجية. نستنتج من الدراسة ان الزيت الطيار لنبات اكليل الجبل له تأثير مضاد للجراثيم بدرجة عالية ضد بكتريا *B. cereus* مقارنة باستخدام مادة النياسين A والذي تسبب في حدوث أضرار أكبر للأعضاء في الكبد والطحال مما يرجح من استخدام الزيوت الطيارة لهذا النبات كمادة حافظة لحفظ اللحوم.

الكلمات المفتاحية: *B. cereus*، منتجات اللحوم، 16 s الرنا الريبوسومي، النياسين، الزيت الطيار ، النياسين اي

INTRODUCTION

Bacillus cereus causes two distinct food poisoning syndromes, characterized, respectively, by emesis and diarrhea. It is most common of which is a highly fulminant posttraumatic endophthalmitis (30). The great majority of research on the pathogenic nature of this organism has been toward identifying and characterizing a diarrheal enterotoxin. The toxin or toxins involved in the diarrheal disease may also be involved in non gastrointestinal infections (6). The research of *B. cereus* with regard food has gained significance in the light of its capability to form endospores have heat resistance and capacity to grow and produce toxins in a wide range of foods (28). Bacteriocins are antimicrobial peptides secreted by certain bacterial strains, which are immune to them, to compete by inhibiting the growth of other bacteria present in their environment. Nisin is a bacteriocin of the lantibiotics group, whose use as a food preservative was approved by the European Union (food additive number E234, EEC, 1983) and the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO), and it is currently the most studied and characterized of all the bacteriocins produced by lactic acid bacteria (LAB). For some food the quantity of Nisin added is about 25 ppm (13). Many reports about lantibiotics extensively subclass of bacteriocins, which includes staphylococin C55 and Nisin A have highly activity against food-borne pathogens (5). However, it was noticed in the recent years that some bacteria have resistance to Nisin A as a result of exposure of cells to high levels of it (17). *Staphylococcus aureus* found to be established resistance easily to nisin due to spontaneous mutation (16). Meat has exerted is an important component of a healthy. It is importance in human nutrition as well as examines some pejorative beliefs about meat consumption. *Rosmarinus officinalis* Rosmary is medicinal plant back to the Lamiaceae family. It used in traditional medicine and in food products as a flavoring agent due to its desirable flavor, antioxidant and antimicrobial effects (14,9). Plant volatile oils have antimicrobial activities; however they limited used in foods due to their strong flavor. In the

latest years, plant extracts used in foods as antioxidants. The rosemary extract was tested against some food spoilage and foodborne pathogenic microorganisms under various conditions of pH, water activity, and temperature (8). Synthetic antimicrobial preservatives were used for many years in the food industry, but their consumption can lead to intoxications, allergies, cancer ...etc. Thus, the search for natural products as new antimicrobial agents derived from animals, plants and microorganisms used in food industry (14). The canned meat products are widely consumed who give concerns about food poisoning caused by pathogens as well as the potential risk of Nisin A. However, although many studies have shown the effect of preservatives on canned food and its harm to consumers, little attention has been paid to evaluating the risks of resistance of contaminated bacteria in canned foods, including canned meat, against Nisin. Thus, this study was focused on isolating *B. cereus* from meat sources that deals with human food poisoning. Furthermore, comparison between the activity of Nisin A and rosemary volatile oil as antibacterial and food preservative material in vitro and in vivo.

MATERIALS AND METHODS

Sampling and culture media

From some markets in Baghdad city, one hundred canned food samples (Fried meat and Grilled meat) were collected. All samples were cultured on mannitol yolk polymyxin agar (MYP), a medium used for the detection, enumeration and isolation of *B. cereus* in food samples then incubated for 24 h at 30°C (11).

Identification of bacteria using 16S rRNA

The 16S rRNA was amplified using the primers 27F (AGAGTTTGATCTTGGCT CAG) and 1492R (TACGGTTACCTTGTT ACGACTT) (29). The PCR tube was contain 12.5 µl of GoTag Green Master Mix (Promega, USA), 1 µM of each primer and 2 ng/µl of template DNA (Wizard Genomic DNA Purification Kit, Promega), the volume was completed to 25 µl with nuclease free water. The PCR reaction was carried out for 5 min at 95°C; 30 cycles of denaturation for 30 sec at 95°C, annealing for 45 sec at 60°C, extension for 1 min at 72°C then final extension for 7 min at 72°C. The PCR product was separate in 1%

agarose gel electrophoresis stained with ethidium bromide and visualized on a UV transilluminator. The amplicons were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation, Korea.

Preparation of rosemary volatile oil and Nisin A

The plant collected from Baghdad University campus-College of science, then dried and finely grounded, with 300g dry plant powder in 1.5 L distilled water for 5h. the volatile oil were extracted by hydro distillation using Clevenger apparatus, and three concentrations 500, 1000 and 2000 µg/ml was prepared with D.W (v/v) and kept at 4°C until use (19, 4). Nisin A (Sigma Nisaplin, Germany) was prepared by dissolved 100 mg from Nisin A powder in 10 ml HCl (0.02 N) to give 10⁴ IU/ml, three concentrations (1300, 650 and 350 IU/ml) were prepared. For sterilization, the solutions were passed throughout 0.45 filters then stored at -20 °C (20).

Determine the inhibitory volatile oil and Nisin A on *B. cereus*

Determination the antimicrobial effect of the Rosemary volatile oil and Nisin A on *B. cereus* planktonic cell using well diffusion assay method was applied according to (10). The concentration give highest diameter of inhibition zone has been chosen for further studies.

Determine the activity of rosemary volatile oil and Nisin A as preservative agents

The fresh meat was obtained from Baghdad market and stored at -15°C. Before the experiments, the samples were thawed at room temperature and cut with a sterile scalpel into small pieces (2.5 cm width×3cm long×1cm high) then washed with sterilized D.W and boiled for 3 h. Finally, the meat pieces were poured into large jars and filled with sterilized D.W then boiled for 30 min (15). The meat pieces were soaked with effective concentration of Rosemary volatile oil and Nisin A solution (2000 µg/ml and 350 IU/ml respectively) for 10 min at room temperature separately. Nine days, after the bacterial through counting of bacterial colony on nutrient agar then compared to meat without adding Rosemary volatile oil and Nisin A (control) every three days (31).

Toxicity assay and histopathological study

Three groups of albino male mice were obtained from Al-Nahran central, aged 5-6 weeks and weighing 20-25 g kept in plastic cages in the animal house of Baghdad Research Center, University of Baghdad. Each group consisted of four mice, group 1 (control) was fed with 0.1 ml of physiological solution via mouth oral syringe while group 2 and 3 were fed orally with effective concentration of Rosemary volatile oil and Nisin A for 14 days respectively. For histopathological study, mice were killed using diethyl ether and vivisection to liver and spleen. The organs were kept in formalin before lab investigation. The mice were left for 10 day for adaptation before the experiments beginning.

Statistical analysis

The results were analyzed using the SPSS IBM version 20. Least significant differences (LSD) test was done to determine the differences between inhibition zone diameters of antimicrobial agent as well as between the colony forming unit count for meat treated with Nisin A, Rosemary volatile oil and control.

RESULTS AND DISCUSSION

The results showed that from 100 meat samples, and screening as *B. cereus* the four isolates were identified on MYP medium and confirmed the identification after the amplification of *16S rRNA* and sequencing the products (1500 bp) as shown in Figure 1. The gene has better efficiency than its counterpart, *16S rRNA* because of faster evolutionary rate which contributes to lesser percentage of gene similarities, even among closely related species and therefore suitable for bacterial species identification (23). The *16S rRNA* gene sequences of three PCR amplicons were compared with the GenBank database using BLASTn in NCBI and found high similarity to the sequences of *Bacillus cereus* ATCC 14579 registered in GenBank with 99% identity. As shows in Figure 2, in inhibitory zone diameters between Nisin A and Rosemary. The results of different Nisin concentrations showed highly significant differences ($P \leq 0.01$). Nisin A inhibitory effects at concentrations 350 and 650 IU/ml had higher diameter while at 1300 µg/ml observed lower diameter. The result agreed with (25) study which reported the

antimicrobial effect of Nisin against *B. cereus* at different concentration during storage. However, the diameter decreased with Nisin A concentration increased may resulting from the

bacteria might be resistant to these high concentrations through unknown mechanism which need further study.

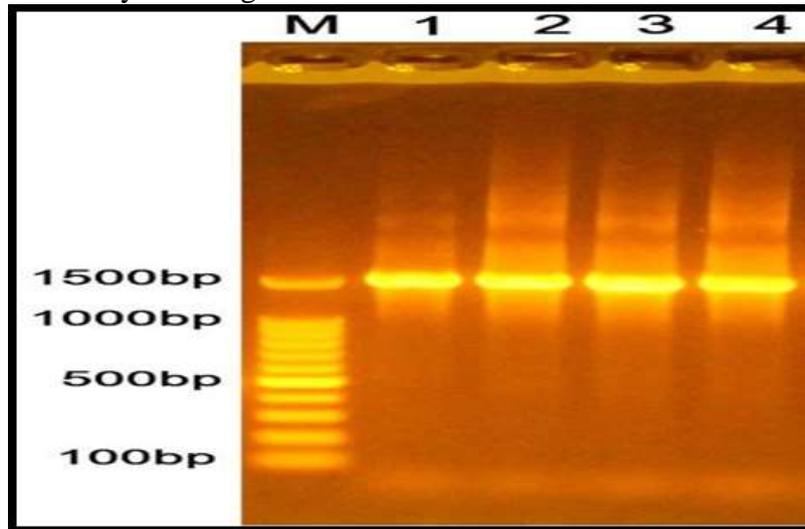


Figure 1. PCR amplicons of the *16S rRNA* (1500 bp) from bacterial genomic DNA stained with ethidium bromide. Lanes 1-4. Unknown bacterial isolates, Lane M. 100 bp DNA ladder. The amplicons was electrophoresis on 1% agarose (1x TBE buffer) at 5 volt/cm² for 90 min

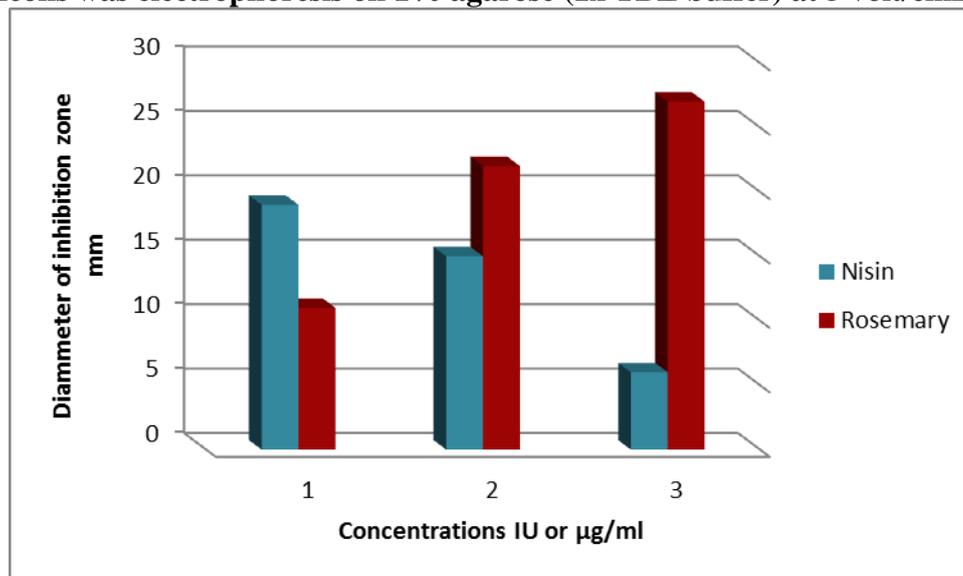


Figure 2. Inhibitory zone diameter of Nisin A concentration range (1-350, 2- 650, 3- 1300 IU/ml) and Rosemary volatile oil at different concentrations (1-500, 2- 1000, 3-2000 µg/ml) on *B. cereus* bacterium

The results of different Rosemary volatile oil concentrations showed highly significant differences ($P \leq 0.01$). The concentrations of the volatile oil (500, 1000 and 2000 µg/ml) used against *B. cereus* showed inhibition zone reached to 11, 22 and 27 mm respectively The rosemary extract as an antioxidant of lipids in foods and antimicrobial against foodborne microorganisms such *Bacillus* spp (1). The volatile oils have strong antibacterial activities against microorganisms due to a high percentage of phenolic secondary metabolites

compounds (2,3,8 24). Many extracts were achieved from ginger roots included water extract, effects some microorganisms such as *Salmonella typhimurium*, *E. coli*, *B. cereus*, *S. aureus* and, *Pseudomonas aeruginosa* (21). The effect of Nisin A and Rosemary on the growth of bacteria on laboratory canned meat are shows in Figure (3) and Table (1). After counting of bacterial colony on nutrient agar every three days, the samples with Rosemary volatile oil was not showed any CFU colony /g after 9 days of preservation while sample with

Nisin A and control observed number of colony between 49 and 45 CFU/g respectively. Moreover, the results in Table 1 show

significant differences ($P \leq 0.05$) in CFU count after 9 day of treating the meat with Rosemary volatile oil in contrast to control and Nisin A.

Table 1. Distribution of *B. cereus* plate count for meat samples

Groups	Distribution of bacterial count (CFU/g)								
	After (3) days Dilution			After (6) days Dilution			After (9) days Dilution		
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Control	25	13	9	37	22	12	49	33	24
Rosemary volatile oil	0	0	0	0	0	0	0	0	0
Nisin A	20	13	9	33	17	11	45	30	18



Figure 3. Preparation of Canned Meat with Nisin A and Rosemary volatile oil at concentrations 350 IU/ml, 2000 µg/ml respectively and control.

Consistent with this result, the average Aerobic plant counts (APCs) for retail cuts of beef samples in the study of Eisel *et al.* (12) were approximately 3 CFU/g. Other results shown combination of equal volume of thyme oil with cinnamon oils lead to reduce the count of *B. cereus* in minced meat 96.09% (27). Henning *et al.* (16) reported that Nisin is unstable on meat which activity decreased rapidly over time, especially at room temperature. It has been suggested that Nisin to meat particles and surfaces may cause loss of Nisin activity. There are problems of using Nisin A for preservation of meat due to low solubility, uneven distribution, and instability on the meat of surface. Therefore, Nisin alone may not be enough to prevent spoilage, as the gram-negative bacteria and Nisin resistance such as lactic acid bacteria are often associated with meat spoilage. The results of toxicity assay showed no death in experimental animal

for all groups of study. In vivo results when vivisection the liver and spleen then preparing the histopathological sections, they were found no harmful effect after using of 350 IU/ml (17.5 mg/kg) of Nisin for 14 days (Figure 4). In contrast to control, 2000 µg/ml (10 mg/kg) Rosemary volatile oil was induced more extensive organ damages such as multifocal liver cell degeneration, necrosis and hemorrhage and multifocal increase of tangible body macrophages in spleen (Figure 5). A common plant grown in many parts of the world (*R. officinalis*), it is used (medicine, as an antispasmodic) (18). On the study the effect of the aqueous extract on overdose consumption of aspartame showed of rosemary extract these defects in the histological architecture of the kidney (26) Marja *et al.*, (22) who found that plants extracts and different types of chromosomal aberrations.

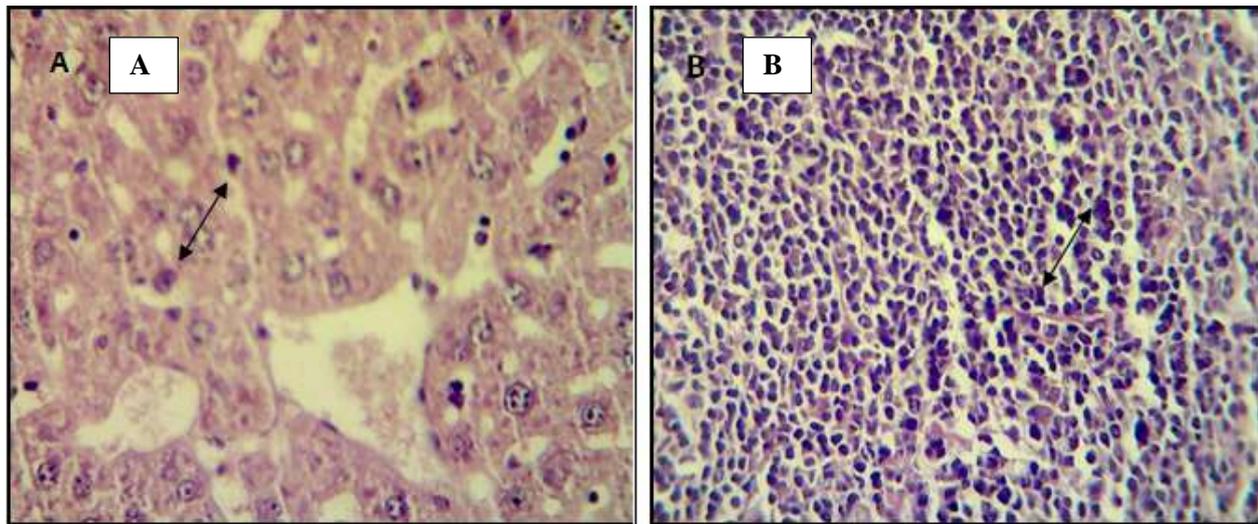


Figure 4. Histopathological section the liver (A) and spleen (B) after treated with Rosemary volatile oil 2000 µg/ml. (A): showed proliferation of kupffer cells with few neutrophils in mild dilated sinusoids. (B): showed moderate proliferation of lymphocytes in periarteriolar sheath. (H and E stain 400X).

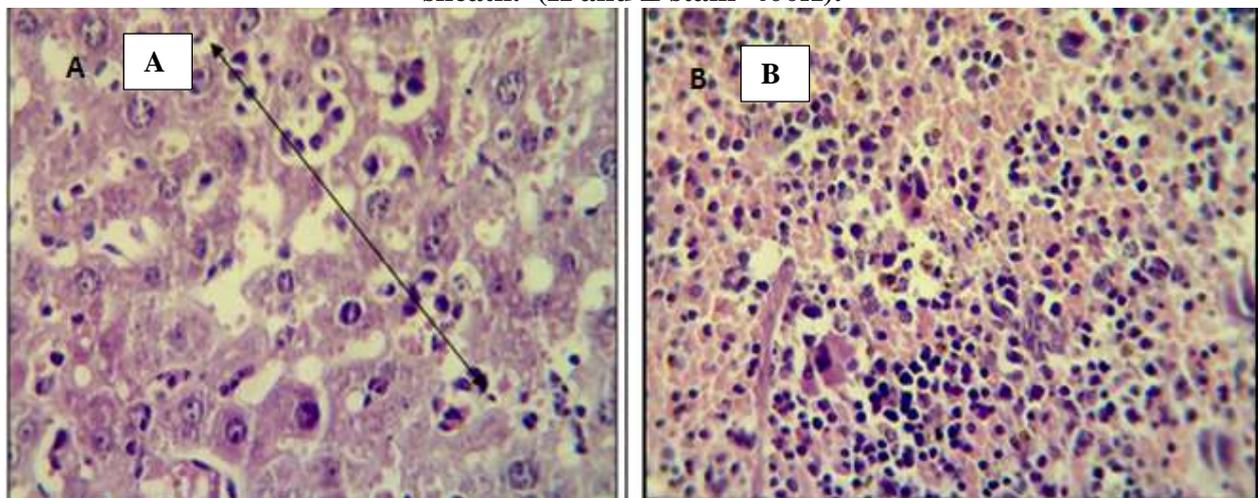


Figure 5. Histopathological section of the liver (A) and spleen (B) after treated with Nicin A 350 IU/ml. (A): showed neutrophils aggregated in dilated congested sinusoids and necrosis of hepatocytes. (B) showed neutrophils infiltration of congested red pulp with depletion of white pulp (H and E stain 400X).

In the light of our results, rosemary volatile oil has proven to be very effective as a preservative substance, as it has shown great effectiveness against the bacteria *B. cereus* isolated from meat compare with using Nisin A at different concentrations which need further studies as preservative agent for meat products.

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